A Relationship Between Thermotolerance and Longevity in *Caenorhabditis elegans*

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Studies of aging in the nematode *Caenorhabditis elegans* have revealed a relationship between stress resistance and the rate of aging: Mutations which extend mean and maximum life-span also confer resistance to thermal stress. We review the molecular genetics of aging in *C. elegans* and introduce methods for obtaining novel mutants which display altered aging rates. We present the use of the "surrogate" phenotype of thermotolerance to develop a selection for novel mutations which slow aging. ***Key words: C. elegans, aging, life-span, thermotolerance, stress resistance. Journal of Investigative Dermatology Symposium Proceedings 3:6–10, 1998***

THE MOLECULAR GENETICS OF NEMATODE AGING

Aging is a highly complex process and is a characteristic of almost all eukaryotes. It is associated with the accumulation of cellular and macromolecular damage but perhaps aging is most accurately defined simply as an increasing probability of death with chronologic age.

Considerable progress has been made recently in identifying processes that limit life-span. The new information is being derived primarily from invertebrate systems by genetic analysis. Invertebrates have been extensively used in aging studies because of their short life-span, relatively simple genetics, and ease with which large populations can be maintained.

The nematode *Caenorhabditis elegans* is an excellent experimental system for the genetic dissection of complex processes such as development and programmed cell death (Riddle *et al*, 1997). This organism is also a very important model system for studying the molecular biology of aging because of the discovery of single-gene mutations that extend life-span (Age mutations; Lithgow, 1996b). *C. elegans* has a life-span of about 20 d under normal lab conditions but the Age mutations can extend life-span by as much as 300% (Larsen *et al*, 1995).

In this paper, we review the recent progress in our understanding of aging in *C. elegans*, we consider the evolutionary origins of aging and we introduce some preliminary findings from our own laboratory that illustrate a relationship between aging and stress response.

C. elegans is a small (1.2 mm) free living, soil dwelling roundworm. It has a 3 d life cycle with four larval stages (L1 to L4) before the final moult into the reproducing adult. The hermaphrodite lives for ™20 d at 20°C and the male usually lives ™18 d (Johnson and Hutchinson, 1993). Poor nutritional conditions or overcrowding lead L1 larvae to develop into an alternative larval stage called the dauer (enduring) larva. The dauer larva is nonfeeding, nonreproducing, stress resistant, and lives four to eight times longer than the adult (Klass and Hirsh, 1976).

Before considering the details of aging processes, we should be aware of the evolutionary origins of aging. Aging, unlike development, is not a tightly regulated, programmed, adaptation. Rather, evolutionary theories of aging suggest natural selection has an *indirect* influence on life-span. This view stems from the observation that very few aged individual animals are to be found in the wild due to high levels of extrinsic hazard resulting from predators and disease. Only in protected environments, such as those experienced by humans or by zoo animals, can aging and the effects of aging be observed. The absence of aged individuals in the wild means that events that happen in late life do not effect Darwinian fitness. Consequently, aging can be seen as the indirect consequence of a lack of selection against intrinsic late-life gene-action. Genes that determine the rate of aging are not likely to be selected due to their influence on aging (Medawar, 1952; Hamilton, 1966; Charlesworth, 1980). In fact, genes that appear to cause detrimental events in late life may actually be maintained in populations due to their beneficial effects in early life (Williams, 1957). All this suggests that there is not likely to be any selection for a life-span-limiting program, but rather that mortality arises as a consequence of selection for other traits.

We can also think of aging in terms of the constraints placed on a biologic system. Each organism has limited resources with which to maintain itself and reproduce itself. Consequently, the genome specifies a life history (characters such as growth rate, developmental time, fertility, life-span) that is optimal – a "best fit" – for that environment. Although it may be possible for a genome to specify an organism that is not subject to age-related deterioration, there would be a concomitant decrease in fitness as other life history traits would be deprived of the resources used in such long-term maintenance. This idea forms the basis of the disposable theory that points to the evolved limitation of maintenance processes due to the maximization of fitness (Kirkwood and Holliday, 1979; Kirkwood and Cremer, 1982; Kirkwood and Franceschi, 1992).

It is important to keep these evolutionary considerations in mind when considering aging processes. One clear prediction of the evolutionary theory is that genes that determine life-span are likely to also influence early life traits. Indeed, the genes that determine life-span in *C. elegans* do influence other early life processes.

Genetic alterations that alter aging rates confer changes in the mean and maximum life-span (Age mutations). The isolation of such mutations in *C. elegans* is made possible by an absence of heterosis for life history traits in this organism (Johnson, 1987; Johnson and Lithgow, 1992; Johnson and Hutchinson, 1993) and, with one possible exception (Brown-Borg *et al*, 1996), all known mutations that confer an increase in metazoan life-span have been identified in this species (Friedman and Johnson, 1988a, b; Van Voorhies, 1992; Kenyon *et al*, 1993; Ishii

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Abbreviation: CR, caloric restriction.

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et al, 1994; Wong *et al*, 1995; Lakowski and Hekimi, 1996). Age mutations lead to increases in life-span due to a decrease in the acceleration of mortality rate with age (Johnson, 1990). In contrast, mutations that cause a decrease in life-span are common (Johnson, 1984) and are unlikely to alter aging rates but rather simply alter initial mortality.

The Age mutations have recently been the subject of classical genetic analysis. Epistatic interactions have allowed for the ordering of genes defined by these mutations into genetic pathways (Lithgow, 1996b). There is evidence of multiple genetic pathways defining life-span (Lakowski and Hekimi, 1996) and one of these pathways partially overlaps with the genetic pathway that determines dauer formation during development (Riddle and Albert, 1997). Five genes in this pathway are known to influence life-span; mutations of *age-1*, *daf-2*, or *daf-28* extended life-span by 70%, 100%, and 30%, respectively, and life-span extension by these mutations requires a wild-type *daf-16* gene (Dorman *et al*, 1995; Larsen *et al*, 1995; Murakami and Johnson, 1996). Mutation of a second gene, *daf-12* in a *daf-2* mutant background, results in life-span extension by 300% (Larsen *et al*, 1995). The *age-1* gene encodes a homolog of mammalian phosphatidylinositol-3-kinase catalytic subunit (PI3K) (Morris *et al*, 1996). The *daf-2* gene (Kenyon *et al*, 1993) encodes a protein that is 35% identical to human insulin receptor and 34% identical to the insulin growth factor 1 receptor (Kimura *et al*, 1997). The *daf-16* gene encodes a protein that is similar to the family of human fork head transcription factors (Ogg *et al*, 1997; Lin *et al*, 1998). The *daf-28* gene is uncloned (Malone *et al*, 1996).

Genetic evidence exists for a second separate life-span pathway, containing the genes *clk-1*, *clk-2*, *clk-3*, and *gro-1* (Lakowski *et al* 1996). This pathway does not influence the formation of dauer larvae (Van Voorhies, 1992; Wong *et al*, 1995; Lakowski and Hekimi, 1996).

The overlap between the dauer formation pathway and one of the aging pathways is highly significant. Despite the fact that the dauer is nonfeeding and nonreproducing, it does share some characteristics with the long-lived *daf-2*, *daf-28*, and *age-1* adults. In particular, both dauers and Age mutant adults are longer lived than normal adult worms and are stress resistant. The simplest interpretation of the overlap is that the Age mutations lead to a partial "dauer-like state" to occur in adult animals and that some dauer-specific processes are activated (Kenyon *et al*, 1993).

It is not known why Age mutations extend life-span. There are two *nonexclusive* models currently being investigated. The first model stems from the observation that many, if not all, Age mutations confer resistance to stress. The model is that the Age genes regulate the expression of stress response genes and the Age mutations lead to elevated levels of stress proteins that subsequently reduce mortality rates (the stress gene model) (Lithgow *et al*, 1995; Johnson *et al*, 1996; Lithgow, 1996a, b). There is considerable evidence consistent with this model, not least that the Age mutations lead to highly significant increases in stress resistance and to elevated levels of antioxidant enzymes and molecular chaperones (Johnson *et al*, 1996). This model is consistent with a vast literature on the accumulation of damaged macromolecules during aging in species ranging from nematode to mammals. One source of this damage is thought to be reactive oxygen species generated by the mitochondrial electron transport chain. The Age mutations may retard aging by the scavenging of reactive oxygen species by antioxidant enzymes and by the maintenance of protein conformation by molecular chaperones.

The second model suggests that Age mutations alter central metabolism and consequently that reactive oxygen species production is decreased (the metabolic model). Two lines of evidence support this model. Firstly, the *clk-1* gene, which when mutated leads to a 100% increase in mean life-span (Lakowski and Hekimi, 1996), is a functional homolog of a yeast gene (*COQ7/CAT5*) that affects the synthesis of the electron carrier ubiquinone (Marbois and Clarke, 1996; Ewbank *et al*, 1997). Loss of this carrier in yeast leads to a respiratory deficiency (Marbois and Clarke, 1996). Such a respiratory defect in *C. elegans* may decrease reactive oxygen species production and consequently slow aging. Support for the metabolic alteration model also comes from the *daf-2* encoded insulin/IGF1 receptor protein and the *age-1*-encoded PI3K. By analogy with mammals, these signaling proteins may regulate glucose uptake and fat and glycogen anabolism through the transcription factor DAF-16 (Kimura *et al*, 1997; Ogg *et al*, 1997; Lin *et al*, 1998).

The stress resistance phenotype associated with Age mutations was first associated with the prototype Age gene, *age-1*. In a test of the oxygen radical theory of aging, two groups showed that the *age-1(hx546)* mutation conferred resistance to the H_2O_2 and the redox cycler, paraquat (Larsen, 1993; Vanfleteren, 1993). Under unstressed conditions the mutant strain also showed elevated levels of Cu/Zn superoxide dismutase and catalase during mid- and late life. Later, the *hx546* mutation was also shown to confer increased thermotolerance (Itt); young adult hermaphrodites grown at 20°C withstood a lethal heat shock at 35°C (Lithgow *et al*, 1994, 1995).

The Itt phenotype was important in two respects: first, two other Age mutations, *spe-26(hc138)* and *daf-2(e1370)*, were shown to confer Itt, suggesting a mechanistic relationship between thermotolerance and aging. Second, it suggested that Age mutations could be isolated by selecting for thermotolerant mutants. We present here evidence that a genetic selection for thermotolerant mutants of *C. elegans* is feasible and that a subset of those new Itt alleles have altered aging rates under nonstressed conditions.

Further evidence for a mechanistic relationship between stress tolerance and longevity came with the discovery that all Age mutations were also resistant to UV radiation (Murakami and Johnson, 1996). UV resistance is an excellent predictor of genetically determined life-span in the nematode (Murakami and Johnson, 1996).

THE IDENTIFICATION OF NOVEL AGE MUTATIONS

For a better understanding of the physiologic processes that limit life-span in this organism, we have undertaken genetic screens for novel Age mutations. Screens based on the extended life-span phenotype are highly labor intensive and therefore we have used a strategy in which we select for mutations that increase stress resistance. The best predictor of extended life-span is UV resistance (Murakami and Johnson, 1996) but we have found that thermotolerance is a suitable phenotype for selection following mutagenesis.

Materials and methods In the preliminary studies we report here, media and standard procedures for the culture of *C. elegans* are as described by Sulston and Hodgkin (1988). Worms were grown and maintained at 20°C until pretreatment or thermal stress. The wild-type Bristol (N2) strain and TJ1052[*age-1(hx546)*II] were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. The age-synchronous populations were established either by moving eggs from a mixed population to a new NGM plate or by placing 50–100 gravid adults onto a small, prespotted NGM plate and allowing them to lay for ~8 h.

The thermotolerance phenotype was scored as previously described (Lithgow *et al*, 1994, 1995) for each of the novel strains. Briefly, 4 d old, gravid adult hermaphrodites were placed on small NGM plates and incubated at 35°C ± 0.5°C. At the indicated times, plates were removed and worms were scored for motility, provoked movement, and pharyngeal pumping. Worms failing to display any of these traits were scored as dead. Mean survival under stress was determined with the Wilcoxon (Gehan) statistic (Lee, 1992).

Dauer formation was assayed by shifting eggs laid at 20°C to 27°C and the emerging worms were scored as either adults or dauers 3 d later. At 27°C, 100% of N2 (wild-type) eggs developed into adults and 100% of TJ1052 [*age-1(hx546)*] eggs developed into dauer larvae.

For complementation analysis, crosses were performed between each strain and mated hermaphrodites were transferred daily and the frequency of male progeny on each day scored. F1 hermaphrodites were subject to further analysis when males comprised 50% of the F1 progeny on a given day.

Survival was analyzed as previously described (Lithgow *et al*, 1995). Briefly, synchronous populations of hermaphrodites were transferred each day during the reproductive period and every 3 d thereafter. Worms were scored for motility, touch-provoked movement, and pharyngeal pumping. Worms failing to display any of these traits were

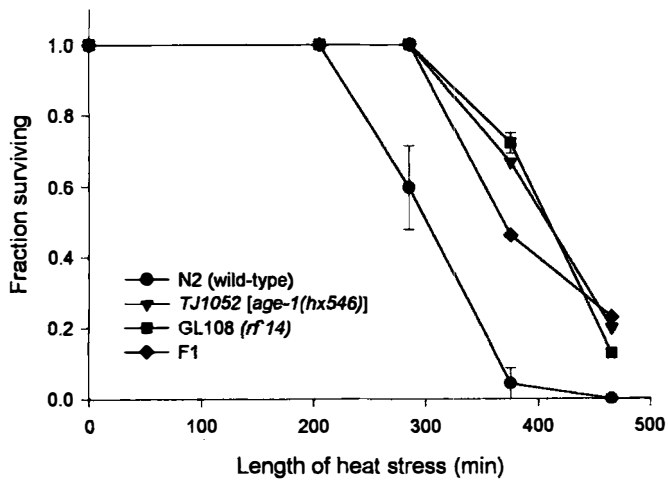

Figure 1. Thermotolerance conferred by a novel mutation. Survival of 4 d old hermaphrodites during 35°C heat shock. The mean survival times (± SEM) were as follows; N2 (wild-type), 343 ± 50 min, TJ1050 [*age-1(hx546)*], 435 ± 45 min, GL108 (*rf14*), F1 [*age-1(hx546)/rf14*], 417 ± 47 min. GL108 is significantly thermotolerant [Wilcoxon (Gehan) survival statistic; $p < 0.0001$], as are the F1 populations demonstrating noncomplementation with the *age-1(hx546)* mutation.

Table I. Novel alleles from a screen for Age mutations

Allele	Thermotolerance	Extended life-span
rf10	✓✓✓✓✓✓	✗✗✗
rf11	✓✓✓✓✓✓	✗
rf12	✓✓✓✓✓✓✗✓✓✓	✓
rf13	✓✓✓✓✓✓✓	✓
*rf14**	✓✓✓✓✓✓✓✓✓✓✓	✓✓✓✓
rf15	✓✓✓✓✓	✗
*rf16**	✓✓✓✓✓✓✓✓✓✓✓✓✓✓✓	✓✓✓✓
rf17	✓✓✓✓✓✓	✓✗✓
rf18	✓✗✓✓	✓
rf19	✓✗✓✓✓	✗
rf20	✓✓✓✓✓	
rf21	✓✓✓✓✓✓✓✓✓✓✓	✓✓

✓, Significantly different from wildtype to $p < 0.0001$. ✗, Not significant. Each tick or cross represents an independent assay on populations of >50 hermaphrodites. * Alleles that confer Daf-c at 27°C.

scored as dead. Mean survival under stress was determined with the Wilcoxon (Gehan) survival statistic (Lee, 1992).

Results We subjected fourth stage larvae (L4) to mutagenesis with ethylmethylsulfonate, which induces small deletions and point mutations. At this life cycle stage, the number of germline nuclei is nearing maximum and exposure to ethylmethylsulfonate results in germline alterations. As previous Age mutations were known to be recessive, we screened the equivalent of 28,000 genomes in F2 worms following mutagenesis. Thirty independent populations of F2 worms were subjected to a lethal heat shock at 35°C and populations were established from the surviving worms. Most of these populations contained fertile worms and thus could be propagated.

This genetic screen resulted in the isolation of a number of novel thermotolerant strains. Clonal lines were established from thermotolerant survivors of the F2 mutant screen and were subject to multiple thermotolerance assays. In all, 11 clonal lines were isolated that were reproducibly Itt (**Fig 1, Table I**). On the assumption that the Itt phenotype results from a single-gene mutation, these lines were preliminarily designated as containing alleles *rf10–rf21*.

Our analysis of the novel Itt alleles has been aimed at assessing the genetic complexity of the Itt phenotype and explaining the relationship between thermotolerance and aging. We have carried out complementation analysis to assign the new alleles either to known Age genes or to new complementation groups. From this analysis, it is clear that

Table II. Thermotolerance of strains carrying wild-type (WT) and mutant alleles of *age-1*

Strain	Survival (Mean ± SD min)
N2 (wild-type)	410 ± 73
GL108 (*rf14*)	456 ± 70
GL110 (*rf16*)	478 ± 55
F1(GL108 × GL110)	470 ± 71*
N2 (wild-type)	343 ± 50
TJ1052 [*age-1(hx546)*]	435 ± 45
GL108 (*rf14*)	440.1 ± 41
F1 (GL108 × TJ1052)	416.7 ± 47*

* Not statistically different from parental strains.

Table III. Dauer formation at 27°C in wild-type, mutant *age-1* strains and in F1 progeny of complementation crosses

Strain	% Dauers (N)
N2 (wild-type)	0% (70)
TJ1052 [*age-1(hx546)*]	100% (46)
GL108 (*rf14*)	100% (76)
GL110 (*rf16*)	100% (81)
F1 (GL110 × GL108)	100% (83)
F1 (GL110 × TJ1052)	100% (56)

at least three genes have been tagged and it appears that at least two of these are novel (data not shown).

Two of the novel thermotolerant strains (containing alleles *rf14* and *rf16*) produce dauers when grown at 27°C, which suggested that these mutations occured in genes in the dauer formation pathway. We carried out crosses with strains carrying the *age-1(hx546)* allele and measured both thermotolerance and dauer formation in the F1 progeny. Both of these alleles show noncomplementation for thermotolerance (**Table II**; **Fig 1**) and for dauer formation (**Table III**).

We have also outcrossed the Itt strains to the parental wild-type strain in order to remove unlinked mutations and to determine the number of mutations leading to the Itt phenotype in any one strain. Of four mutant strains backcrossed, the Itt phenotype assorts as a single gene in three and the fourth displays a more complex inheritance with the likelihood being that this strain carries two or more Itt mutations (data not shown).

Survival and mortality analyses of the novel Itt strains were undertaken in synchronously aging hermaphrodite populations of either 50 or 100 worms, as previously described (Brooks *et al*, 1994). Of the 11 Itt alleles, seven appear to increase mean and maximum life-span (**Table I**). Like the *age-1(hx546)* allele, the new *age-1* alleles (*rf14* and *rf16*) exhibit extended life-span and reduced age-specific mortality rates (**Fig 2**).

FUTURE STUDIES IN NEMATODE AGING GENETICS

The Age mutations of *C. elegans* clearly demonstrate the genetic determination of life-span and show that this complex process can be analyzed using genetic tools. Those Age genes cloned (*age-1*, *daf-2*, and *clk-1*) illustrate that such genetic analysis can yield valuable information on the nature of aging processes. We consider the isolation and analysis of many mutations that extend life-span essential to identify key influences of the insulin signaling pathway, and other pathways, on aging. To this end, we have utilized an associated phenotype of all of the Age mutations, i.e., Itt, to identify novel Age mutations.

In a search for recessive mutations that alter tolerance to thermal stress, we find that over half the alleles conferring thermotolerance also confer extended life-span. This finding has implications for the genetic analysis of stress tolerance and aging.

Many of the Itt alleles are also long-lived, suggesting that processes leading to thermotolerance in the face of acute thermal stress also extend life-span under nonstressed conditions. The fact that aging rates and stress tolerance are determined by overlapping sets of genes suggests a tight mechanistic relationship and prompts us to suggest a role for

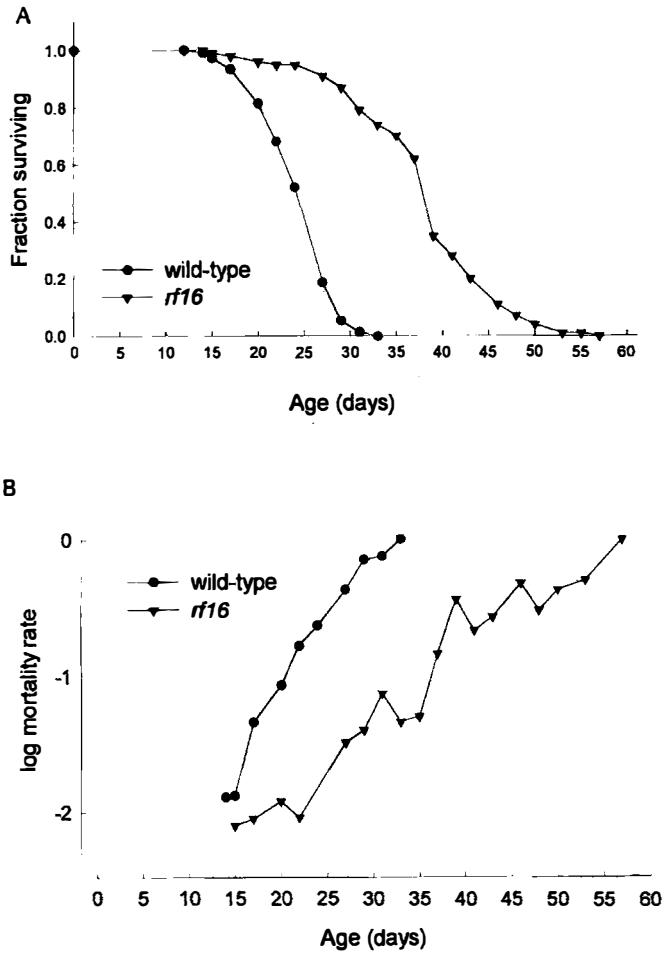

Figure 2. Survival and mortality analyses of a novel thermotolerant strain. (*A*) The strain carrying *rf16* exhibited extended life-span [Wilcoxon (Gehan) survival statistic; $p < 0.0001$]. The mean life-spans (± SEM) were as follows; N2 (wild-type) ± days, *rf16* ± days. (*B*) Mortality is the fraction of worms dying over the following 2 d. Mortality is significantly less in the *rf16* carrying strain over the life-span (log rank test; $p < 0.0001$).

stress response proteins, such as heat shock proteins, in modulating rates of aging. Aging is characterized in many systems by an accumulation of macromolecular damage (Martin *et al*, 1996). We have proposed a model whereby molecular chaperones act during aging to counteract an accumulation conformationally altered protein that would otherwise aggregate, interfere with other cellular processes, and lead to increasing mortality (Lithgow, 1996a).

There are a number of observations that are consistent with this model. There is a clear correlation between thermotolerance and life-span that goes beyond the Age mutations; subjecting worm cultures to mild-transient thermal stress results in both acquired thermotolerance and extended life-span (Lithgow *et al*, 1995). We note numerous studies that show the accumulation of noncovalently altered protein (reviewed in Lithgow, 1996a). Consistent with this is the observation that older animals are more refractory to stress and fail to induce molecular chaperone genes during stress to the same levels as young animals (Lithgow *et al*, 1994). Also notable is the observation that molecular chaperone gene products accumulate in the absence of environmental stress in old fruit flies, suggesting that these chaperones are specifically required in late life (Wheeler *et al*, 1996).

A recent test of the hypothesis that molecular chaperones influence aging rates has been made by Tatar *et al* (1997). In experiments that utilize transgenic lines of *Drosophila* that carry additional copies of the gene encoding the inducible *hsp-70* gene, they show an effect of this molecular chaperone on aging; induction of this transgene is associated with reduction in mortality rates. This is the clearest indication of an important mechanistic relationship between the stress response genes and aging rates.

On the basis of the stress gene model, we would predict that the novel Itt mutations described here will display elevated levels of molecular chaperones during stress and aging. We would also predict that manipulation of the levels of specific molecular chaperones in adult worms would lead to changes in aging rates. We are currently testing these predictions.

The study described here shows that Age mutations can be identified using an associated phenotype and thus be found faster than with genetic screens based on the life-span phenotype. We envisage that a combination of genetic screens based on stress tolerance would allow for the identification and cloning of many of the genes that co-ordinately regulate the response and define life-span.

As new information emerges on the nature of the processes that influence worm aging, we begin to look for similarities to aging processes in other systems. Caloric restriction (CR) is the most extensively studied experimental manipulation of aging rate in rodents. CR is the reduction in the total dietary energy component that can result in life-span extension of up to 40% (Masoro and Austad, 1996). As discussed previously, the nematode *daf-2* gene shares extensive homology with the insulin receptor and the insulin-like growth factor receptor (Kimura *et al*, 1997). CR is known to cause a decrease of insulin-like growth factor-1 and insulin-like growth factor-1 binding proteins (Breese *et al*, 1991) in rodents. One is tempted to speculate that similar physiologic alterations occur in both *daf-2* nematodes and CR-treated rodents. Likewise, CR rodents exhibit elevated stress tolerance and expression of molecular chaperone genes in response to acute stress, which again parallels the nematode Age mutations (Heydari *et al*, 1993). It has also been noted (Kimura *et al*, 1997) that Ames dwarf mice, which have a pituitary deficiency, also exhibit an extended life-span (Brown-Borg *et al*, 1996). These mice exhibit very low insulin-like growth factor-1 levels and again this may mirror metabolic changes similar to those in the *daf-2* Age mutants.

The emerging parallel between aging processes in highly divergent species encourage the idea that studying invertebrate systems may indeed lead to an understanding of mammalian aging and hence age-related disease.

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